[0026] It is fu-rthermore recognized that an important way to optimize an enzymatic process is through the modification and improvement of the pivotal catalytic enzyme. For example, a transgenic plant can be formed that is comprised of an expression system for expressing a phytase molecule. It is appreciated that by attempting to improve factors that are not directly related to the activity of the expressed molecule proper, such as the expression level, only a finite - and potentially insufficient - level of optimization may be maximally achieved. Accordingly, there is also a need for obtaining molecules with improved characteristics.

[0050] **Figures 1a and 1b** shows the nucleotide and deduced amino acid sequences the enzyme of the present invention. Sequencing was performed using a 378 automated DNA sequencer (Applied Biosystems, Inc.).

[0059] The present invention provides purified a recombinant phytase enzyme, shown in Figures 1a and 1b. Additionally, the present invention provides isolated nucleic acid molecules (polynucleotides) which encode for the mature enzyme having the deduced amino acid sequence of Figures 1a and 1b. The invention also provides modified phytase sequences as shown in Figure 8 and SEQ ID NO:9 and 10.

[0065] The terms "fragment", "derivative" and "analog" when referring to the enzyme of Figures 1a and 1b comprise a enzyme which retains at least one biological function or activity that is at least essentially same as that of the reference enzyme. Furthermore, the terms "fragment", "derivative" or "analog" are exemplified by a "pro-form" molecule, such as a low activity proprotein that can be modified by cleavage to produce a mature enzyme with significantly higher activity.

[0078] The present invention provides a "nucleic acid construct" or alternatively a "nucleotide construct" or alternatively a "DNA construct". The term "construct" is used herein to describe a molecule, such as a polynucleotide (*e.g.*, a phytase polynucleotide) may optionally be chemically bonded to one or more additional molecular moieties, such as a vector, or parts of a vector. In a specific - but by no means limiting - aspect, a nucleotide construct is exemplified by a DNA expression ~~DNA expression~~ constructs suitable for the transformation of a host cell.

[0085] As used herein, the term "reagent" includes phytase molecules of the instant invention. Preferably, such phytase molecules catalyze the hydrolysis of phytate to inositol and free phosphate with release of minerals from the phytic acid complex. An exemplary phytase

molecule is a phytase derived from *Escherichia coli* B. This exemplary enzyme is shown in Figures 1a and 1b, SEQ ID NO:2. Additionally, as used herein, the term "reagent" includes substrate reagents molecules of the instant invention, such as phytate molecules. Preferably, such phytate molecules are found in foodstuffs, potential foodstuffs, byproducts of foodstuffs (both *in vitro* byproducts and *in vivo* byproducts, *e.g.* ex vivo reaction products and animal excremental products), precursors of foodstuffs, and any other source of phytate.

[0093] The present invention provides purified a recombinant enzyme that catalyzes the hydrolysis of phytate to inositol and free phosphate with release of minerals from the phytic acid complex. An exemplary purified enzyme is a phytase derived from *Escherichia coli* B. This exemplary enzyme is shown in Figures 1a and 1b, SEQ ID NO:2.

[0094] The enzymes of the present invention include, in addition to an enzyme of Figures 1a and 1b (in particular the mature enzyme), polypeptides having sequences that are "substantially identical" to the sequence of a phytase polypeptide, such as one of SEQ ID 1.

[0096] The phytase polypeptide included in the invention can have the amino acid sequences of the phytase shown in Figures 1a and 1b (SEQ ID NO:2). Phytase polypeptides, such as those isolated from *E. coli* B , can be characterized by catalyzing the hydrolysis of phytate to inositol and free phosphate with the release of minerals from the phytic acid complex.

[0106] The present invention further relates to an enzyme which has the deduced amino acid sequence of Figures 1a and 1b, as well as analogs, derivatives, and fragments of such enzyme.

[0107] An analog, derivative, or fragment of the enzyme of Figures 1a and 1b may be (a) one in which one or more of the amino acid residues are substituted with an amino acid residue which is not encoded by the genetic code, or (b) one in which one or more of the amino acid residues includes a substituent group, or (c) one in which the mature enzyme is fused with another compound, such as a compound to increase the half-life of the enzyme (for example, polyethylene glycol), or (d) to provide a label or a tag, such as a 6xHis tag or a green fluorescent protein tag, (e) one in which the additional amino acids are fused to the mature enzyme, such as a leader or secretory sequence or a sequence which is employed for purification of the mature enzyme or a proprotein sequence. Such analogs, derivatives, and fragments are deemed to be within the scope of those skilled in the art from the teachings herein.

[0115] In accordance with an aspect of the present invention, there are provided isolated nucleic acid molecules (polynucleotides) which encode for the mature enzyme having the deduced amino acid sequence of Figures 1a and 1b.

[0117] In accordance with another aspect of the present invention, there is provided an isolated polynucleotide encoding an exemplary enzyme of the present invention (SEQ ID NO:1) comprising the DNA of Figures 1a and 1b.

[0120] The polynucleotide of the present invention may be in the form of DNA which DNA includes cDNA, genomic DNA, and synthetic DNA. The DNA may be double-stranded or single-stranded, and if single stranded may be the coding strand or non-coding (anti-sense) strand. The coding sequence which encodes the mature enzyme may be identical to the coding sequences shown in Figures 1a and 1b and/or that of the deposited clone (SEQ ID NO:1), or may be a different coding sequence which coding sequence, as a result of the redundancy or degeneracy of the genetic code, encodes the same mature enzyme as the DNA of Figures 1a and 1b (*e.g.*, SEQ ID NO:1).

[0121] The polynucleotide which encodes for the mature enzyme of Figures 1a and 1b (*e.g.*, SEQ ID NO:2) may include, but is not limited to: only the coding sequence for the mature enzyme; the coding sequence for the mature enzyme and additional coding sequence such as a leader sequence or a proprotein sequence; the coding sequence for the mature enzyme (and optionally additional coding sequence) and non-coding sequence, such as introns or non-coding sequence 5' and/or 3' of the coding sequence for the mature enzyme.

[0122] The present invention further relates to variants of the hereinabove described polynucleotides which encode for fragments, analogs and derivatives of the enzyme having the deduced amino acid sequence of Figures 1a and 1b (*e.g.*, SEQ ID NO:2). The variant of the polynucleotide may be a naturally occurring allelic variant of the polynucleotide or a non-naturally occurring variant of the polynucleotide.

[0123] Thus, the present invention includes polynucleotides encoding the same mature enzyme as shown in Figures 1a and 1b as well as variants of such polynucleotides which variants encode for a fragment, derivative or analog of the enzyme of Figures 1a and 1b. Such nucleotide variants include deletion variants, substitution variants and addition or insertion variants.

[0124] As hereinabove indicated, the polynucleotide may have a coding sequence which is a naturally occurring allelic variant of the coding sequence shown in Figures 1a and 1b. As known in the art, an allelic variant is an alternate form of a polynucleotide sequence which may have a substitution, deletion or addition of one or more nucleotides, which does not substantially alter the function of the encoded enzyme.

[0141] A man-made ~~genes~~ gene produced using the invention can also serve as a substrate for recombination with another nucleic acid. Likewise, a man-made gene pathway produced using the invention can also serve as a substrate for recombination with another nucleic acid. In a preferred instance, the recombination is facilitated by, or occurs at, areas of homology between the man-made intron-containing gene and a nucleic acid with serves as a recombination partner. In a particularly preferred instance, the recombination partner may also be a nucleic acid generated by the invention, including a man-made gene or a man-made gene pathway. Recombination may be facilitated by or may occur at areas of homology that exist at the one (or more) artificially introduced intron(s) in the man-made gene.

[0152] In ~~a~~ another embodiment, the invention includes a method for producing a hybrid polynucleotide from at least a first polynucleotide and a second polynucleotide. The invention can be used to produce a hybrid polynucleotide by introducing at least a first polynucleotide and a second polynucleotide which share at least one region of partial sequence homology into a suitable host cell. The regions of partial sequence homology promote processes which result in sequence reorganization producing a hybrid polynucleotide. The term "hybrid polynucleotide", as used herein, is any nucleotide sequence which results from the method of the present invention and contains sequence from at least two original polynucleotide sequences. Such hybrid polynucleotides can result from intermolecular recombination events which promote sequence integration between DNA molecules. In addition, such hybrid polynucleotides can result from intramolecular reductive reassortment processes which utilize repeated sequences to alter a nucleotide sequence within a DNA molecule.

[0163] In another aspect, methods can be used to generate novel polynucleotides encoding biochemical pathways from one or more operons or gene clusters or portions thereof. For example, bacteria and many eukaryotes have a coordinated mechanism for regulating genes whose products are involved in related processes. The genes are clustered, in structures referred

to as "gene clusters," on a single chromosome or immediately adjacent to one another and are transcribed together under the control of a single regulatory sequence, including a single promoter which initiates transcription of the entire cluster. Thus, a gene cluster is a group of adjacent genes that are either identical or related, usually as to their function. An example of a biochemical pathway encoded by gene clusters ~~are~~is polyketides. Polyketides are molecules which are an extremely rich source of bioactivities, including antibiotics (such as tetracyclines and erythromycin), anti-cancer agents (daunomycin), immunosuppressants (FK506 and rapamycin), and veterinary products (monensin). Many polyketides (produced by polyketide synthases) are valuable as therapeutic agents. Polyketide synthases are multifunctional enzymes that catalyze the biosynthesis of an enormous variety of carbon chains differing in length and patterns of functionality and cyclization. Polyketide synthase genes fall into gene clusters and at least one type (designated type I) of polyketide synthases have large size genes and enzymes, complicating genetic manipulation and *in vitro* studies of these genes/proteins.

[0165] Therefore, in a one embodiment, the invention relates to a method for producing a biologically active hybrid polypeptide and screening such a polypeptide for enhanced activity by:

1) introducing at least a first polynucleotide in operable linkage and a second polynucleotide in operable linkage, said at least first polynucleotide and second polynucleotide sharing at least one region of partial sequence homology, into a suitable host cell;
2) growing the host cell under conditions which promote sequence reorganization resulting in a hybrid polynucleotide in operable linkage;
3) expressing a hybrid polypeptide encoded by the hybrid polynucleotide;
4) screening the hybrid polypeptide under conditions which promote identification of enhanced biological activity; and
5) isolating the ~~a~~ polynucleotide encoding the hybrid polypeptide.

[0167] As representative examples of expression vectors which may be used there may be mentioned viral particles, baculovirus, bacteriophage 1 insertion vectors or replacement vectors, phage, plasmids, phagemids, cosmids, fosmids, bacterial artificial chromosomes (BAC), viral DNA (*e.g.*, vaccinia, adenovirus, foul pox virus, pseudorabies and derivatives of SV40),

P1-based artificial chromosomes (PAC), yeast plasmids, yeast artificial chromosomes (YAC), and any other vectors specific for specific hosts of interest (such as bacillus, aspergillus and yeast). Thus, for example, the DNA may be included in any one of a variety of expression vectors for expressing a polypeptide. Such vectors include chromosomal, nonchromosomal and synthetic DNA sequences. Large numbers of suitable vectors are known to those of skill in the art, and are commercially available. The following vectors are provided by way of example; Bacterial: pQE vectors (Qiagen), pBluescript® plasmids, pNH vectors, (lambda-ZAP® vectors (Stratagene); ptrc99a, pKK223-3, pDR540, pRIT2T (Pharmacia); Eukaryotic: pXT1, pSG5 (Stratagene), pSVK3, pBPV, pMSG, pSVLSV40 (Pharmacia). However, any other plasmid or other vector may be used so long as they are replicable and viable in the host. Low copy number or high copy number vectors may be employed with the present invention.

[0219] The following vectors are provided by way of example; Bacterial: pQE70, pQE60, pQE-9 (Qiagen), pBluescript II® (Stratagene); pTRC99a, pKK223-3, pDR540, pRIT2T (Pharmacia); Eukaryotic: pXT1, pSG5 (Stratagene) pSVK3, pBPV, pMSG, pSVLSV40 (Pharmacia). However, any other plasmids or other vectors may be used as long as they are replicable and viable in the host.

[0241] Examples of a variant phytase polynucleotide sequence include sequences substantially as set forth in SEQ ID NO:7, wherein the polynucleotide has a nucleotide sequence as set forth in a) SEQ ID NO:9; b) SEQ ID NO:9 wherein all Ts are Us (RNA); wherein the expression of the phytase-encoding nucleic acid leads to the production of said substantially pure phytase enzyme; and c) SEQ ID NO:7, wherein 389 is G; 390 is A; nucleotide 437 is T; 438 is G; 439 is G; 470 is C; ~~427~~72 is T; 476 is T; 477 is G; 478 is T; 689 is G; 690 is A; 691 is G; 728 is T; 729 is A; 730 is T; 863 is T; 864 is G; 1016 is G, or any combination thereof. More specifically, with respect to part c), the invention provides a nucleotide sequence substantially identical to SEQ ID NO:7, and having a modified nucleotide sequence selected from nucleotide 389 is G and 390 is A (SEQ ID NO:5); nucleotide 437 is T, 438 is G and 439 is G (SEQ ID NO:6); 470 is C and 472 is T; 476 is T, 477 is G, and 478 is T; 689 is G, 690 is A and 691 is G; 728 is T, 729 is A, and 730 is T; 863 is T and 864 is G; 1016 is G, or any combination thereof.

[0246] An oligonucleotide of the invention can include a portion of a phytase polynucleotide, including, for example, a sequence substantially identical to that of SEQ ID NO:7, except

wherein nucleotide wherein 389 is G; 390 is A; nucleotide 437 is T; 438 is G; 439 is G; 470 is C; ~~427~~72 is T; 476 is T; 477 is G; 478 is T; 689 is G; 690 is A; 691 is G; 728 is T; 729 is A; 730 is T; 863 is T; 864 is G; 1016 is G, or wherein the oligonucleotide contains a combination of such substitutions with respect to SEQ ID NO:7. Thus, as disclosed herein, the oligonucleotide can be any length and can encompass one or more of the above mutations.

[0254] The choice of a vector will also depend on the size of the polynucleotide sequence and the host cell to be employed in the methods of the invention. Thus, the vector used in the invention can be plasmids, phages, cosmids, phagemids, viruses (e.g., retroviruses, parainfluenzavirus, herpesviruses, reoviruses, paramyxoviruses, and the like), or selected portions thereof (e.g., coat protein, spike glycoprotein, capsid protein). For example, cosmids and phagemids are typically used where the specific nucleic acid sequence to be analyzed or modified is large because these vectors are able to stably propagate large polynucleotides. Cosmids and phagemids are particularly suited for the expression or manipulation of the phytase polynucleotide~~a~~s of SEQ ID NO:1 or 7 or a mutant phytase polynucleotide as in SEQ ID NO:9.

[0271] In one aspect of the invention, a method for producing a~~n~~ phytase enzyme, such as ~~those~~ that shown in Figure~~s~~ 1, is provided. The method includes growing a host cell which contains a polynucleotide encoding the enzyme (e.g., SEQ ID NO: 1, 7 or 9), under conditions which allow the expression of the nucleic acid, and optionally isolating the enzyme encoded by the nucleic acid. Methods of culturing the host cell are described in the Examples and are known by those of skill in the art.

[0275] The introduction of gene ~~constucts~~ constructs into plants can be achieved using several technologies including transformation with *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes*. Non-limiting examples of plant tissues that can be transformed thusly include protoplasts, microspores or pollen, and explants such as leaves, stems, roots, hypocotyls, and cotyls. Furthermore, DNA can be introduced directly into protoplasts and plant cells or tissues by microinjection, ~~electriporation~~ electroporation, particle bombardment, and direct DNA uptake.

[0276] Proteins may be produced in plants by a variety of expression systems. For instance, the use of a constitutive promoter such as the 35S promoter of Cauliflower Mosaic Virus (Guilley *et al.*, 1982) is serviceable for the accumulation of the expressed protein in virtually all

organs of the transgenic plant. Alternatively, the use of promoters that are highly tissue-specific and/or stage-specific are serviceable for this invention (Higgins, 1984; Shotwell, 1989) in order to bias expression towards desired tissues and/or towards a desired stage of development. Further details relevant to the expression in plants of the phytase molecules of the instant invention are disclosed, for example, in USPN 5,770,413 (Van Ooijen *et al.*) and USPN 5,593,963 (Van Ooijen *et al.*), although these references do not teach the inventive molecules of the instant application and instead teach the use of fungal phytases.

[0277] In sum, it is relevant to this invention that a variety of means can be used to achieve the recombinant expression of phytase in a transgenic plant or plant part. Such ~~a~~ transgenic plants and plant parts are serviceable as sources of recombinantly expressed phytase, which can be added directly to phytate-containing sources. Alternatively, the recombinant plant-expressed phytase can be extracted away from the plant source and, if desired, purified prior to contacting the phytase substrate.

[0280] Several techniques are available for the introduction of the expression construct containing the phytase-encoding DNA sequence into the target plants. Such techniques include but are not limited to transformation of protoplasts using the calcium/polyethylene glycol method, electroporation and microinjection or (coated) particle bombardment (Potrykus, 1990). In addition to these so-called direct DNA transformation methods, transformation systems involving vectors are widely available, such as viral vectors (*e.g.* from the Cauliflower Mosaic Cirus (CaMV)) and bacterial vectors (*e.g.* from the genus Agrobacterium)) (Potrykus, 1990). After selection and/or screening, the protoplasts, cells or plant parts that have been transformed can be regenerated into whole plants, using methods known in the art (Horsch *et al.*, 1985). The choice of the transformation and/or regeneration techniques is not critical for this invention.

[0300] In a particular aspect, this invention provides a method of catalyzing *in vivo* and/or *in vitro* reactions using seeds containing enhanced amounts of enzymes. The method comprises adding transgenic, non-wild type seeds, preferably in a ground form, to a reaction mixture and allowing the enzymes in the seeds to increase the rate of reaction. By directly adding the seeds to the reaction mixture the method provides a solution to the more expensive and cumbersome process of extracting and purifying the enzyme. Methods of treatment are also provided whereby an organism lacking a sufficient supply of an enzyme is administered the enzyme in the

form of seeds from one or more plant species, preferably transgenic plant species, containing enhanced amounts of the enzyme. Additional details regarding this approach are in the public literature and/or are known to the skilled artisan. In a particular non-limiting exemplification, such publicly available literature includes USPN 5,543,576 (Van Ooijen *et al.*) and USPN 5,714,474 (Van Ooijen *et al.*), although these references do not teach the inventive molecules of the instant application and instead teach the use of fungal phytases.

[0301] In a particular non-limiting aspect, the instant phytase molecules are serviceable for generating recombinant digestive system life forms (or microbes or flora) and for the administration of said recombinant digestive system life forms to animals. Administration may be optionally performed alone or in combination with other enzymes &/or with other life forms that can provide enzymatic activity in a digestive system, where said other enzymes and said life forms may be ~~may~~ recombinant or otherwise. For example, administration may be performed in combination with xylanolytic bacteria.

[0302] In a non-limiting aspect, the present invention provides a method for steeping corn or sorghum kernels in warm water containing sulfur dioxide in the presence of an enzyme preparation comprising one or more phytin-degrading enzymes, preferably in such an amount that the phytin present in the corn or sorghum is substantially degraded. The enzyme preparation may comprise phytase and/or acid phosphatase and optionally other plant material degrading enzymes. The steeping time may be 12 to 18 hours. The steeping may be interrupted by an intermediate milling step, reducing the steeping time. In a preferred embodiment, corn or sorghum kernels are steeped in warm water containing sulfur dioxide in the presence of an enzyme preparation including one or more phytin-degrading enzymes, such as phytase and acid phosphatases, to eliminate or greatly reduce phytic acid and the salts of phytic acid. Additional details regarding this approach are in the public literature and/or are known to the skilled artisan. In a particular non-limiting exemplification, such publicly available literature includes USPN 4,914,029 (Caransa *et al.*) and EP 0321004 (Vaara *et al.*), although these references do not teach the inventive molecules of the instant application.

[0308] In a non-limiting aspect, the present invention provides a method to obtain an absorbefacient capable of promoting the absorption of minerals including ingested calcium without being digested by gastric juices or intestinal juices at a low cost. In a preferred

embodiment, said mineral absorbefacient contains a partial hydrolysate of phytic acid as an active ingredient. Preferably, a partial hydrolyzate of the phytic acid is produced by hydrolyzing the phytic acid or its salts using novel phytase molecules of the instant invention. The treatment with said phytase molecules may occur either alone &/or in a combination treatment (to inhibit or to augment the final effect), and is followed by inhibiting the hydrolysis within a range so as not to liberate all the phosphate radicals. Additional details regarding this approach are in the public literature and/or are known to the skilled artisan. In a particular non-limiting exemplification, such publicly available literature includes JP 04270296 (Hoshino), although references in the publicly available literature do not teach the inventive molecules of the instant application.

[0309] In a non-limiting aspect, the present invention provides a method (and products therefrom) to produce an enzyme composition having an additive or preferably a synergistic phytate hydrolyzing activity; said composition comprises novel phytase molecules of the instant invention and one or more additional reagents to achieve a composition that is serviceable for a combination treatment. In a preferred embodiment, the combination treatment of the present invention is achieved with the use of at least two phytases of different position specificity, *i.e.* any combinations of 1-, 2-, 3-, 4-, 5-, and 6-phytases. By combining phytases of different position specificity, an additive or synergistic effect is obtained. Compositions such as food and feed or food and feed additives comprising such phytases in combination are also included in this invention as are processes for their preparation. Additional details regarding this approach are in the public literature and/or are known to the skilled artisan. In a particular non-limiting exemplification, such publicly available literature includes WO9 830681 (Ohmann *et al.*), although references in the publicly available literature do not teach the use of the inventive molecules of the instant application.

[0310] In another preferred embodiment, the combination treatment of the present invention is achieved with the use of an acid phosphatase having phytate hydrolyzing activity at a pH of 2.5, in a low ratio corresponding to a pH 2.5:5.0 activity profile of from about 0.1:1.0 to 10:1, preferably of from about 0.5:1.0 to 5:1, more preferably still of from about 0.8:1.0 to 3:1, and more preferably still of from about 0.8:1.0 to 2:1. Said enzyme composition preferably displays a higher synergetic phytate hydrolyzing efficiency through thermal treatment. Said enzyme

composition is serviceable in the treatment of foodstuffs (drinkable and solid food, feed and fodder products) to improve phytate hydrolysis. Additional details regarding this approach are in the public literature and/or are known to the skilled artisan. In a particular non-limiting exemplification, such publicly available literature includes USPN 5,554,399 (Vanderbeke *et al.*) and USPN 5,443,979 (Vanderbeke *et al.*), although these references do not teach the use of the inventive molecules of the instant application, but rather teach the use of fungal (in particular *Aspegillus*) phytases.

[0311] In a non-limiting aspect, the present invention provides a method (and products therefrom) to produce compositions comprised of the instant novel phytate-acting enzyme in combination with one or more additional enzymes that act on polysaccharides. Such polysaccharides can be selected from the group consisting of arabinans, fructans, fucans, galactans, galacturonans, glucans, mannans, xylans, levan, fucoidan, carrageenan, galactocarolose, pectin, pectic acid, amylose, pullulan, glycogen, amylopectin, cellulose, carboxylmethylcellulose, hydroxypropylmethylcellulose, dextran, pustulan, chitin, agarose, keratan, chondroitin, dermatan, hyaluronic acid, alginic acid, and polysaccharides containing at least one aldose, ketose, acid or amine selected from the group consisting of erythrose, threose, ribose, arabinose, xylose, lyxose, allose, altrose, glucose, mannose, gulose, idose, galactose, talose, erythrulose, ribulose, xylulose, psicose, fructose, sorbose, tagatose, glucuronic acid, gluconic acid, glucaric acid, galacturonic acid, mannuronic acid, glucosamine, galactosamine and neuraminic acid.

[0312] In a particular aspect, the present invention provides a method (and products therefrom) to produce compositions having a synergistic phytate hydrolyzing activity comprising one or more novel phytase molecules of the instant invention, a cellulase (including preferably, but not exclusively, a xylanase), optionally a protease, and optionally one or more ~~additonal~~ additional reagents. In preferred embodiments, such combination treatments are serviceable in the treatment of foodstuffs, wood products, such as paper products, and as cleansing solutions and solids.

[0315] In another non-limiting exemplification, the instant phytase molecules are serviceable for generating life forms that can provide at least one enzymatic activity - either alone or in combination treatments - in the treatment of digestive systems of organisms. Particularly

relevant organisms to be treated include non-ruminant organisms. Specifically, it is appreciated that this approach may be performed alone or in combination with other biological molecules (for example, xylanases) to generate a recombinant host that expresses a plurality of biological molecules. It is also appreciated that the administration of the instant phytase molecules &/or recombinant hosts expressing the instant phytase molecules may be performed either alone or in combination with other biological molecules, &/or life forms that can provide enzymatic activities in a digestive system - where said other enzymes and said life forms may be ~~may~~ recombinant or otherwise. For example, administration may be performed in combination with xylanolytic bacteria.

[0318] Additional details regarding this approach are in the public literature and/or are known to the skilled artisan. In a particular non-limiting exemplification, such publicly available literature includes USPN 5,624,678 (Bedford *et al.*), USPN 5,683,911 (Bodie *et al.*), USPN 5,720,971 (Beauchemin *et al.*), USPN 5,759,840 (Sung *et al.*), USPN 5,770,012 (Cooper), USPN 5,786,316 (Baeck *et al.*), USPN 5,817,500 (Hansen *et al.*), and journal articles (Jeffries, 1996; Prade, 1996; Bayer *et al.*, 1994; Duarte *et al.*, 1994; Hespell & Whitehead, 1990; Wong *et al.*, 1988), although these reference do not teach the inventive phytase molecules of the instant application, nor do they all teach the addition of phytase molecules in the production of foodstuffs, wood products, such as paper products, and as cleansing solutions and solids. In contrast, the instant invention teaches that phytase molecules – preferably the inventive phytase molecules of the instant application - may be added to the reagent(s) disclosed in order to obtain preparations having an additional phytase activity. Preferably, said reagent(s) and the additional phytase molecules and will not inhibit each other, more preferably said reagent(s) and the additional phytase molecules will have an overall additive effect, and more preferably still said reagent(s) and the additional phytase molecules will have an overall synergistic effect.

[0319] In a non-limiting aspect, the present invention provides a method (and products therefrom) for enhancement of phytate phosphorus utilization and treatment and prevention of tibial dyschondroplasia in animals, particularly poultry, by administering to animals a feed composition containing a hydroxylated vitamin D_3 derivative. The vitamin D_3 derivative is preferably administered to animals in feed containing reduced levels of calcium and phosphorus for enhancement of phytate phosphorus utilization. Accordingly, the vitamin D_3 derivative is

preferably administered in combination with novel phytase molecules of the instant invention for further enhancement of phytate phosphorus utilization. Additional details regarding this approach are in the public literature and/or are known to the skilled artisan. In a particular non-limiting exemplification, such publicly available literature includes USPN 5,516,525 (Edwards *et al.*) and USPN 5,366,736 (Edwards *et al.*), USPN 5,316,770 (Edwards *et al.*) although these references do not teach the inventive molecules of the instant application.

[0320] In a non-limiting aspect, the present invention provides a method (and products therefrom) to obtain foodstuff that 1) comprises phytin that is easily absorbed and utilized in a form of inositol in a body of an organism; 2) that is capable of reducing phosphorus in excrementary matter; and 3) that is accordingly useful for improving environmental pollution. Said foodstuff is comprised of an admixture of a phytin-containing grain, a lactic acid-producing microorganism, and a novel phytase molecule of the instant invention. In a preferred embodiment, said foodstuff is produced by compounding a phytin- containing grain (preferably, *e.g.* rice bran) with an effective microbial group having an acidophilic property, producing lactic acid, without producing butyric acid, free from pathogenicity, and a phytase. Examples of an effective microbial group include *e.g. Streptomyces sp.* (ATCC 3004) belonging to the group of actinomyces and *Lactobacillus sp.* (IFO 3070) belonging to the group of lactobacilli. Further, a preferable amount of ~~addition of~~ an effective microbial group to be added is 0.2 wt. % in terms of bacterial body weight based on a grain material. Furthermore, the amount ~~of the addition~~ of the phytase to be added is preferably 1-2 wt. % based on the phytin in the grain material. Additional details regarding this approach are in the public literature and/or are known to the skilled artisan. In a particular non-limiting exemplification, such publicly available literature includes JP 08205785 (Akahori *et al.*), although references in the publicly available literature do not teach the inventive molecules of the instant application.

[0321] In a non-limiting aspect, the present invention provides a method for improving the solubility of vegetable proteins. More specifically, the invention relates to methods for the solubilization of proteins in vegetable protein sources, which methods comprise treating the vegetable protein source with an ~~efficient~~ effective amount of one or more phytase enzymes – including phytase molecules of the instant invention - and treating the vegetable protein source with an ~~efficient~~ effective amount of one or more proteolytic enzymes. In another aspect, the

invention provides animal feed additives comprising a phytase and one or more proteolytic enzymes. Additional details regarding this approach are in the public literature and/or are known to the skilled artisan. In a particular non-limiting exemplification, such publicly available literature includes EP 0756457 (WO 9528850 A1) (Nielsen and Knap), although references in the publicly available literature do not teach the inventive molecules of the instant application.

[0323] In a non-limiting aspect, the present invention provides a method (and products thereof) to activate inert phosphorus in soil and/or compost, to improve the utilization rate of a nitrogen compound, and to suppress propagation of pathogenic molds by adding three reagents, phytase, saponin and chitosan, to the compost. In a non-limiting embodiment the method can comprise treating the compost by 1) adding phytase-containing microorganisms in media – preferably recombinant hosts that overexpress the novel phytase molecules of the instant invention - *e.g.* at 100 ml media/100 kg wet compost; 2) alternatively also adding a phytase-containing plant source - such as wheat bran - *e.g.* at 0.2 to 1 kg/100 kg wet compost; 3) adding a saponin-containing source - such as peat, mugworts and yucca plants – *e.g.* at 0.5 to 3.0g/kg ; 4) adding chitosan-containing materials – such as pulverized shells of shrimps, crabs, etc. – *e.g.* at 100 to 300g/kg wet compost. In another non-limiting embodiment, recombinant ~~sources~~ forms of the three reagents, phytase, saponin, and chitosan, are used. Additional details regarding this approach are in the public literature and/or are known to the skilled artisan. In a particular non-limiting exemplification, such publicly available literature includes JP 07277865 (Toya Taisuke), although references in the publicly available literature do not teach the inventive molecules of the instant application.

[0326] The invention facilitates production of phytase-specific nucleic acid probes. Methods for obtaining such probes can be designed based on the amino acid sequences shown in Figures 1a and 1b. The probes, which can contain at least 12, *e.g.*, at least 15, 25, 35, 50, 100, or 150 nucleotides, can be produced using any of several standard methods (see, *e.g.*, Ausubel *et al.*, *supra*). For example, preferably, the probes are generated using PCR amplification methods. In these methods, primers are designed that correspond to phytase-conserved sequences (see Figures 1a and 1b), which can include phytase-specific amino acids, and the resulting PCR product is used as a probe to screen a nucleic acid library, such as a cDNA library.

[0327] This invention can be used to isolate nucleic acid sequences substantially similar to the isolated nucleic acid molecule encoding an phytase enzyme disclosed in Figures 1a and 1b (SEQ ID NO:1). Isolated nucleic acid sequences are substantially similar if: (i) they are capable of hybridizing under stringent conditions, hereinafter described, to SEQ ID NO:1; or (ii) they encode a phytase polypeptide as set forth in SEQ ID NO:2 due to the degeneracy of the genetic code (*e.g.*,degenerate to SEQ ID NO:1).

[0337] This invention provides enzymes, as well as fragments, other derivatives, and analogs thereof, and cells expressing them that can be used as an immunogen to produce antibodies thereto. These antibodies can be, for example, polyclonal or monoclonal antibodies. The present invention also includes chimeric, single chain, and humanized antibodies, as well as Fab fragments, or the product of ~~an~~ Fab expression library. Various procedures known in the art may be used for the production of such antibodies and fragments.

[0338] Antibodies generated against the enzymes corresponding to a sequence of the present invention can be obtained by direct injection of the enzymes into an animal or by administering the enzymes to an animal, preferably a nonhuman. The antibody so obtained will then bind the enzymes itself. In this manner, even a sequence encoding only a fragment of the enzymes can be used to generate antibodies binding the whole native enzymes. Such antibodies can then be used to isolate the enzyme from cells expressing that enzyme.

[0343] As is mentioned above, antigens that can be used in producing phytase-specific antibodies include phytase polypeptides, *e.g.*, any of the phytase shown in Table 3 or~~Figures 1~~ polypeptide fragments thereof. The polypeptide or peptide used to immunize an animal can be obtained by standard recombinant, chemical synthetic, or purification methods. As is well known in the art, in order to increase immunogenicity, an antigen can be conjugated to a carrier protein. Commonly used carriers include keyhole limpet hemocyanin (KLH), thyroglobulin, bovine serum albumin (BSA), and tetanus toxoid. The coupled peptide is then used to immunize the animal (*e.g.*, a mouse, a rat, or a rabbit). In addition to such carriers, well known adjuvants can be administered with the antigen to facilitate induction of a strong immune response.

[0346] This invention also includes additonal uses of fragments of the phytase polypeptides that retain at least one phytase-specific activity or epitope. Phytase activity can be assayed by

examining the catalysis of phytate to inositol and free phosphate. Such fragments can easily be identified by comparing the sequences of phytases found ~~in~~ to Figures 1a and 1b.

[0347] In a non-limiting exemplification, a phytase polypeptide fragment containing, *e.g.*, at least 8-10 amino acids can be used as an immunogen in the production of phytase-specific antibodies. The fragment can contain, for example, an amino acid sequence that is conserved in phytases, and this amino acid sequence can contain amino acids that are conserved in phytases. In another non-limiting exemplification~~;~~, the above-described phytase fragments can be used in immunoassays, such as ELISAs, to detect the presence of phytase-specific antibodies in samples.

[0348] Various methods to make the transgenic animals of the subject invention can be employed. Generally speaking, three such methods may be employed. In one such method, an embryo at the pronuclear stage (a "one cell embryo") is harvested from a female and the transgene is microinjected into the embryo, in which case the transgene will be chromosomally integrated into both the germ cells and somatic cells of the resulting mature animal. In another such method, embryonic stem cells are isolated and the transgene is incorporated therein by electroporation, plasmid transfection or microinjection, followed by reintroduction of the stem cells into the embryo where they colonize and contribute to the germ line. Methods for microinjection of mammalian species is described in U.S. Pat. No. 4,873,191. In yet another such method, embryonic cells are infected with a retrovirus containing the transgene whereby the germ cells of the embryo have the transgene chromosomally integrated therein. When the animals to be made transgenic are avian, because avian fertilized ova generally go through cell division for the first twenty hours in the oviduct, microinjection into the pronucleus of the fertilized egg is problematic due to the inaccessibility of the pronucleus. Therefore, of the methods to make transgenic animals described generally above, retrovirus infection is preferred for avian species, for example as described in U.S. Pat No. 5,162,215. If micro-injection is to be used with avian species, however, a published procedure by Love *et al.*, (Biotechnology, 12, Jan 1994) can be utilized whereby the embryo is obtained from a sacrificed hen approximately two and one-half hours after the laying of the previous laid egg, the transgene is microinjected into the cytoplasm of the germinal disc and the embryo is cultured in a host shell until maturity. When the animals to be made transgenic are bovine or porcine, microinjection can be hampered by the opacity of the ova thereby making the nuclei difficult to identify by traditional differential

interference-contrast microscopy. To overcome this problem, the ova can first be centrifuged to segregate the pronuclei for better visualization.

[0349] The "non-human animals" of the invention include bovine, porcine, ovine and avian animals (*e.g.*, cow, pig, sheep, chicken). The "transgenic non-human animals" of the invention are produced by introducing "transgenes" into the germline of the non-human animal. Embryonal target cells at various developmental stages can be used to introduce transgenes. Different methods are used depending on the stage of development of the embryonal target cell. The zygote is the best target for micro-injection. The use of zygotes as is target for gene transfer has a major advantage in that in most cases the injected DNA will be incorporated into the host gene before the first cleavage (Brinster *et al.*, Proc. Natl. Acad. Sci. USA 82:4438-4442, 1985). As a consequence, all cells of the transgenic non-human animal will carry the incorporated transgene. This will in general also be reflected in the efficient transmission of the transgene to offspring of the founder since 50% of the germ cells will harbor the transgene.

[0358] Thus, the present invention includes methods for increasing the phosphorous uptake in the transgenic animal and/or decreasing the amount of ~~polltant~~ pollutant in the manure of the transgenic organism by about 15%, typically about 20%, and more typically about 20% to about 50%.

[0361] For example, ~~a~~ nucleic acid constructs of the present invention will comprise nucleic acid molecules in a form suitable for uptake into target cells within a host tissue. The nucleic acids may be in the form of bare DNA or RNA molecules, where the molecules may comprise one or more structural genes, one or more regulatory genes, antisense strands, strands capable of triplex formation, or the like. Commonly, the nucleic acid construct will include at least one structural gene under the transcriptional and translational control of a suitable regulatory region. More usually, nucleic acid constructs of the present invention will comprise nucleic acids incorporated in a delivery vehicle to improve transfection efficiency, wherein the delivery vehicle will be dispersed within larger particles comprising a dried hydrophilic excipient material.

[0369] Hard, biocompatible objects consumed (or otherwise introduced) and presente~~d~~ in the gizzard provide a useful vector for delivery of various enzymatic, chemical, therapeutic and antibiotic agents. These hard substances have a life span of a few hours to a few days and are

passed after a period of time. Accordingly, the invention provides coated, impregnated (*e.g.*, impregnated matrix and membranes) modified dietary aids for delivery of useful digestive or therapeutic agents to an organism. Such dietary aids include objects which are typically ingested by an organism to assist in digestion within the gizzard (*e.g.*, rocks or grit). The invention provides biocompatible objects that have coated thereon or impregnated therein agents useful as a digestive aid for an organism or for the delivery of a therapeutic or medicinal agent or chemical.

[0373] It is contemplated that a number of different biocompatible substances may be ingested or otherwise provided to the same organism simultaneously, or in various combinations (*e.g.*, one material before the other). In addition, the biocompatible substance may be designed for slow passage through the digestive tract. For example, large or fatty substances tend to move more slowly through the digestive tract, accordingly, a biocompatible material having a large size to prevent rapid passing in the digestive tract can be used. Such large substances can be a combination of non-biodegradable and biodegradable substances. For example, a small non-biodegradable substance can be encompassed by a biodegradable substance such that over a period of time the biodegradable portion will be degraded allowing the non-biodegradable portion to pass through the digestive trace. In addition, it is recognized that any number of flavorings can be provided to the biocompatible substance to assist in consumption.

[0384] Dry compositions may be spray dried compositions, in which case the composition need not contain anything more than the enzyme in a dry form. Usually, however, dry compositions are so-called granulates which may readily be mixed with a food or feed components, or more preferably, form a component of a pre-mix. The particle size of the enzyme granulates preferably is compatible with that of the other components of the mixture. This provides a safe and convenient means of incorporating enzymes into animal feed. Preferably, the granulates are biocompatible and more preferably ~~they~~ the biocompatible granulates are non-biodegradable.

[0385] Agglomeration granulates coated by an enzyme can be prepared using agglomeration techniques in a high shear mixer, Absorption granulates are prepared by having cores of a carrier material to absor~~p~~b/be coated by the enzyme. Preferably, the carrier material is a biocompatible non-biodegradable material that simulates the role of stones or grit in the gizzard of an animal.

Typical filler materials used in agglomeration techniques include salts, such as disodium sulphate. Other fillers are kaolin, talc, magnesium ~~aluminium~~ aluminum silicate and cellulose ~~fibres~~ fibers. Optionally, binders such as dextrins are also included in agglomeration granulates. The carrier materials can be any biocompatible material including biodegradable and non-biodegradable materials (*e.g.*, rocks, stones, ceramics, various polymers). Optionally, the granulates are coated with a coating mixture. Such mixture comprises coating agents, preferably hydrophobic coating agents, such as hydrogenated palm oil and beef tallow, and if desired other additives, such as calcium carbonate or kaolin.

[0387] In ~~a~~ one embodiment, the dietary aid compositions of the invention additionally comprises an effective amount of one or more feed enhancing enzymes, in particular feed enhancing enzymes selected from the group consisting of α-galactosidases, β-galactosidases, in particular lactases, other phytases, β-glucanases, in particular endo-β-1,4-glucanases and endo-β-1,3(4)-glucanases, cellulases, xylosidases, galactanases, in particular arabinogalactan endo-1,4-β-galactosidases and arabinogalactan endo-1,3-β-galactosidases, endoglucanases, in particular endo-1,2-β-glucanase, endo-1,3-α-glucanase, and endo-1,3-β-glucanase, pectin degrading enzymes, in particular pectinases, pectinesterases, pectin lyases, polygalacturonases, arabinanases, rhamnogalacturonases, rhamnogalacturonan acetyl esterases, rhamnogalacturonan-α-rhamnosidase, pectate lyases, and α-galacturonisidases, mannanases, β-mannosidases, mannan acetyl esterases, xylan acetyl esterases, proteases, xylanases, arabinoxylanases and lipolytic enzymes such as lipases, phospholipases and cutinases.

[0390] Examples of other specific uses of the phytase of the invention are~~is~~ in soy processing and in the manufacture of inositol or derivatives thereof.

[0391] The invention also relates to a method for reducing phytate levels in animal manure, wherein the animal is fed a dietary aid containing an effective amount of the phytase of the invention. As stated in the beginning of the present application, one important effect thereof is to reduce the phosphate pollution in~~of~~ the environment.

[0393] When the dietary aid is a porous particle, such particles are typically impregnated by a substance with which ~~it is desired to release slowly~~ to form a slow release particle. Such slow release particles may be prepared not only by impregnating the porous particles with the substance it is desired to release, but also by first dissolving the desired substance in the first

dispersion phase. In this case, slow release particles prepared by the method in which the substance to be released is first dissolved in the first dispersion phase are also within the scope and spirit of the invention. The porous hollow particles may, for example, be impregnated by a slow release substance such as a medicine, agricultural chemical or enzyme. In particular, when porous hollow particles impregnated by an enzyme are made of a biodegradable polymers, the particles themselves may be used as an agricultural chemical or fertilizer, and they have no adverse effect on the environment. In one embodiment, the porous particles are magnetic in nature.

[0394] The porous hollow particles may be used as a bioreactor support, in particular an enzyme support. Therefore, it is advantageous to prepare the dietary aid utilizing a method for ~~of~~ ~~a~~ slow release, for instance by encapsulating the enzyme of agent in a microvesicle, such as a liposome, from which the dose is released over the course of several days, preferably between about 3 to 20 days. Alternatively, the agent (*e.g.*, an enzyme) can be formulated for slow release, such as incorporation into a slow release polymer from which the ~~dosage of~~ agent (*e.g.*, enzyme) is slowly released over the course of several days, for example from 2 to 30 days and can range up to the life of the animal.

[0396] Also within the scope of the invention is the use of a phytase of the invention during the preparation of food or feed preparations or additives, *i.e.*, the phytase exe~~e~~rts its phytase activity during the manufacture only and is not active in the final food or feed product. This aspect is relevant for instance in dough making and baking. Accordingly, phytase or recombinant yeast expressing phytase can be impregnated in, on or through ~~a~~ magnetic carriers, distributed in the dough or food medium, and retrieved by magnets.

[0397] The dietary aid of the invention may be administered alone to animals in a~~n~~ biocompatible (*e.g.*, a biodegradable or non-biodegradable) carrier or in combination with other digestion additive agents. The dietary aid of the invention thereof can be readily administered as a top dressing or by mixing them directly into animal feed or provided separate from the feed, by separate oral dosage, by injection or by transdermal means or in combination with other growth related edible compounds, the proportions of each of the compounds in the combination being dependent upon the particular organism or problem being addressed and the degree of response desired. It should be understood that the specific dietary dosage administered in any given case

will be adjusted in accordance with the specific compounds being administered, the problem to be treated, the condition of the subject and the other relevant facts that may modify the activity of the effective ingredient or the response of the subject, as is well known by those skilled in the art. In general, either a single daily dose or divided daily dosages may be employed, as is well known in the art.

[0398] If administered separately from the animal feed, forms of the dietary aid can be prepared by combining them with non-toxic pharmaceutically acceptable edible carriers to make either immediate release or slow release formulations, as is well known in the art. Such edible carriers may be either solid or liquid such as, for example, corn starch, lactose, sucrose, soy flakes, peanut oil, olive oil, sesame oil and propylene glycol. If a solid carrier is used, the dosage form of the compounds may be tablets, capsules, powders, troches or lozenges or top dressing as micro-dispersable forms. If a liquid carrier is used, soft gelatin capsules, or syrup or liquid suspensions, emulsions or solutions may be the dosage form. The dosage forms may also contain adjuvants, such as preserving, stabilizing, wetting or emulsifying agents, solution promoters, etc. They may also contain other therapeutically valuable substances.

[0399] Thus, ~~a~~ significant advantages of the invention include for example, 1) ease of manufacture of the active ingredient loaded biocompatible compositions; 2) versatility as it relates to the class of polymers and/or active ingredients which may be utilized; 3) higher yields and loading efficiencies; and 4) the provision of sustained release formulations that release active, intact active agents *in vivo*, thus providing for controlled release of an active agent over an extended period of time. In addition, another advantage is due to the local delivery of the agent with-in the digestive tract (*e.g.*, the gizzard) of the organism. As used herein the phrase "contained within" denotes a method for formulating an agent into a composition useful for controlled release, over an extended period of time ~~of the agent~~.

[0400] In the sustained-release or slow release compositions of the invention, an effective amount of an agent (*e.g.*, an enzyme or antibiotic) will be utilized. As used herein, sustained release or slow release refers to the gradual release of an agent from a biocompatible material, over an extended period of time. The sustained release can be continuous or discontinuous, linear or non-linear, and this can be accomplished using one or more biodegradable or non-biodegradable compositions, drug loadings, selection of excipients, or other modifications.

However, it is to be recognized that it may be desirable to provide for a "fast" release composition, that provides for rapid release once consumed by the organism. It is also to be understood that ~~by~~ "release" does not necessarily mean that the agent is released from the biocompatible carrier. Rather in one embodiment, the slow release encompasses slow activation or continual activation of an agent present on the biocompatible composition. For example, a phytase need not be released from the biocompatible composition to be effective. In this embodiment, the phytase is immobilized on the biocompatible composition.

[0401] The animal feed may be any protein-containing organic meal normally employed to meet the dietary requirements of animals. Many of such protein-containing meals are typically primarily composed of corn, soybean meal or a corn/soybean meal mix. For example, typical commercially available products fed to fowl include Egg Maker Complete, a poultry feed product of Land O'Lakes AG Services, as well as Country Game & Turkey Grower a product of ~~Agwa~~Agway, Inc. (see also The Emu Farmer's Handbook by Phillip Minnaar and Maria Minnaar). Both of these commercially available products are typical examples of animal feeds with which the present dietary aid and/or the enzyme phytase may be incorporated to reduce or eliminate the amount of supplemental phosphorus, zinc, manganese and iron intake required in such compositions.

[0409] This invention also provides for the isolation and use of phytase molecules (nucleic acids and phytase enzymes encoded thereby) from all other strains of E. coli (whether virulent or non-virulent, including K12, W, C), as well as all bacteria. These include all known species and strains belonging to:

Thermotogales
Green Nonsulfur Bacteria
Cyanobacteria & chloroplasts
Low G+C Gram-Positive Bacteria
Fusobacteria
High G+C Gram-Positive Bacteria
Gytophaga/Flexibacter/Bacteroides group
Fibrobacteria
Spriochaetes
Planctomyces/Chlamydia group

Purple bacteria (Proteobacteria), including the following subdivisions:
    Delta & Epsilon, including:

Desulfuromonas acetoxidans
Desulfosarcina variabilis
Bdellovibrio stolpii
Nannocystis exedens
Stigmatella aurantiaca
Myxococcus xanthus
Desulfovibrio desulfuricans
Thiovulum sp.
Campylobacter jejuni
Wolinella succinogenes
Helicobacter pylori

Alpha, including:

Methylobacterium extorquens
Beijerinckia indica
Hyphomicrobium vulgare
Rhodomicrobium vannieli
Agrobacterium tumefaciens
Brucella abortus
Rochalimaea quintana
Rhodopseudomonas marina subsp. agilis
Zea mays – mitochondrion
Rickettsia rickettsii
Ehrlichia risticii
Wolbachia pipientis
Anaplasma marginale
Erythrobacter longus
Rhodospirillum salexigens
Rhodobacter capsulatus
Azospirillum lipoferum
Rhodospirillum rubrum

Gamma, including:

Ectothiorhodospira shaposhnikovii
Chromatium vinosum
Methylomonas methanica
Cardiobacterium hominis
Xanthomonas maltophilia
Coxiella burnetii
Legionella pneumophila subsp. pneumophila

Oceanospirillum linum
Acinetobacter calcoaceticus
Pseudomonas aeruginosa
Haemophilus influenzae
Vibrio parahaemolyticus
Proteus vulgaris
Erwinia carotovora
Echerichia coli, including:

Beta, including:

Eikenella corrodens
Neisseria gonorrhoeae
Vitreoscilla stercoraria
Chromobacterium violaceum
Alcaligenes faecalis
Rubrivivax gelatinosus
Pseudomonas testosteroni
Nitrosomonas europae
Spirillum volutans

Such phytase molecules can be isolated from these bacteria by known methods, including library screening methods, *e.g.* expression screening, hybridization methods, PCR (*e.g.* see Sam~~m~~brook, 1989).

At page 135, line 22:

EXAMPLE ~~1~~2
THERMAL TOLERANCE ASSAY

The wild type appA from E. coli (strain K12) and a mutagenized version designated 819PH59 (SEQ ID NO:9 and 10) were expressed in E. coli and purified to homogeneity. In the thermal tolerance assay, 100 uL of 0.01 mg/mL of protein in 100 mM MOPS / pH 7.0 was heated to the indicated incubation temperature for 5 minutes in an RJ research thermocycler. Upon completion of the 5 minutes at the incubation temperature, the samples were cooled to 4°C and incubated on ice. An activity assay was run using 40 uL of the enzyme solution in 1.5 mL of 100 mM NaOAc / 4 mM phytate / pH 4.5 at 37 °C. Aliquots of 60 uL were withdrawn at 2 minute intervals and added to 60 uL of the color developer/Stop solution of the TNO assay. Clearly, the

modified enzyme, SEQ ID NO:10, containing 8 amino acid changes, is tolerant to temperatures greater than the wild-type enzyme. (Ssee Figure 3.)

At page 136, line 3:

Example ~~2~~3

Stability of Phytase Enzyme in Simulated Digestibility Conditions

The percent residual activities (based on initial rates) of the in vitro digested E.coli K12 and the nonglycosylated 819pH59 phytase were plotted verses time. A standard concentration of simulated gastric fluid containing 2 mg/ml NaCl, 6 M HCl and 3.2 mg/mL pepsin was prepared as described. The pH of the solution was about 1.4 and was not adjusted. The in vitro digestibility assay was performed by adding 1:4 (vol:vol) of phytase to digestion solution and immediately incubating at 37~~o~~° C to initiate the digestion reaction. Aliquots of the digestion reaction mixture were removed at various time intervals and assayed for residual phytase activity using the TNO assay. Each of the assays was performed at least twice. An exponential curve with the equation y = Ae-kt was fit to the data. The half lives of the proteins were determined using the equation t 1/2 = ln 2/ k . The half-life of the E.coli K12 phytase was only 2.7 ± 0.2 minutes while the nonglycosylated 819pH59 phytase had a half-life of 8.4 ± 1.1 minutes. Therefore, the mutations in the wildtype E.coli K12 phytase enhanced the stability of the enzyme under simulated in vitro digestibility conditions.

(See Figure 4.)

At page 136, line 20:

Example ~~3~~4
Expression Host Comparisons

The GSSM DNA construct from 819PH59 was inserted into E. coli, P. pastoris, and S. pombe for expression. The expressed proteins were purified to homogeneity. In the thermal tolerance assay, 100 uL of 0.01 mg/mL of protein in 100 mM MOPS, pH 7.0 was heated to the indicated incubation temperature for 5 minutes in an RJ research thermocycler. Upon completion of the 5 minutes at the incubation temperature, the samples were cooled to 4°C and incubated on ice. An activity assay was run using 40 uL of the enzyme solution in 1.46 mL of 100 mM NaOAc / 4

mM phytate / pH 4.5 at 37 °C. Aliquots of 60 uL were withdrawn at 2 minute intervals and added to 60 uL of the color developer/Stop solution of the TNO assay. (Ssee Figure 5.).

At page 137, line 1:

Example 45

The percent residual activities (based on initial rates) of the *in vitro* digested 819pH59 phytase expressed in various expression hosts were plotted verses time. The 819pH59 phytase was expressed in *E.coli* (nonglycosylated), as well as in *S.pombe* and *P. pastoris* (glycosylated). A standard concentration of simulated gastric fluid containing 2 mg/ml NaCl, 6 M HCl and 3.2 mg/mL pepsin was prepared as described in the S.O.P. The pH of the solution was about 1.4 and was not adjusted. The *in vitro* digestibility assay was performed by adding 1:4 (vol:vol) of phytase to digestion solution and immediately incubating at 37 °C to initiate the digestion reaction. Aliquots of the digestion reaction mixture were removed at various time intervals and assayed for residual phytase activity using the TNO assay. Each of the assays was performed in triplicate. An exponential curve with the equation $y = Ae^{-kt}$ was fit to the data. The half lives of the proteins were determined using the equation $t_{1/2} = \ln 2/k$. The half-life of the nonglycosylated 819pH59 phytase expressed in *E.coli* was 8.4 ± 1.1 minutes while the glycosylated 819pH59 phytase expressed in *S. pombe* had a half-life of 10.4 ± 0.9 minutes and the same phytase expressed in *P. pastoris* had a half-life of 29.2 ± 6.7 mins. Therefore, the glycosylation of the 819pH59 phytase enhanced the stability of the enzyme under simulated *in vitro* digestibility conditions. (Ssee fFigure 6.)

At page 138, line 15:

Bird *et al.* *Plant Mol Biol* 11:651, 1988..

At page 140, line 15:

Gordon-Kamm WJ, Spencer TM, Mangano ML, Adams TR, Daines RJ, Start WG, O'Brien JV, Chambers SA, Adams Jr. WR, Willets NG, Rice TB, Mackey CJ, Krueger RW, Kausch AP, Lemaux PG. *Plant Cell* 2:603, 1990..